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Titration of Antibodies to Salmonella O Antigens by Enzyme-Linked Immunosorbent Assay

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A new type of immunoassay (enzyme-linked immunosorbent assay, ELISA) has been used for the detection of antibodies against *Salmonella* O antigens. The results show that ELISA is specific and highly sensitive. When compared with the Widal reaction, passive hemagglutination, and quantitative precipitation, ELISA was shown to be more sensitive. In addition, immunoglobulin G and immunoglobulin M antibodies were both detected approximately as efficiently with ELISA.

Detection and quantitation of antibodies to microbial antigens in sera from patients with symptomatic or subclinical infectious diseases constitutes an important problem. For the diagnosis of typhoid and paratyphoid fever, determination of serum titers is an important diagnostic tool. The antibodies are generally detected by tube agglutination (Widal reaction [17]) or by passive hemagglutination tests (14). The Widal reaction is insensitive, and nonspecific reactions are frequently observed (spontaneous agglutination, etc.). Both the passive hemagglutination method and the Widal reaction have an important disadvantage in that antibodies of the immunoglobulin M (IgM) class are preferentially detected. In fact, the passive hemagglutination test may be 10 to 100 times more sensitive for IgM antibodies than for immunoglobulin G (IgG) antibodies, when compared on a weight basis.

This communication deals with the application of a recently developed "enzyme-linked immunosorbent assay" (ELISA) (2, 3, 4) for the detection of *Salmonella* O antibodies. To obtain qualitative and quantitative comparisons between ELISA and other methods, antisera were also tested against homologous antigens in Widal agglutination, passive hemagglutination, and in quantitative precipitation tests. Antisera fractionated in different immunoglobulin classes were also compared in ELISA, passive hemagglutination tests, and quantitative precipitation tests.

MATERIALS AND METHODS

Bacterial strains. For immunization and preparation of antigen, the following *Salmonella* strains were used: *S. typhimurium* LT2, *S. paratyphi* A variant Durazzo, *S. strasbourg*, and *S. senftenberg*. The bacteria were cultivated in complex broth medium as described earlier (8). The yield was 4 to 6 g of bacteria per liter of culture.

Isolation of LPS. The bacteria were harvested by centrifugation, killed by gamma irradiation and washed twice in phosphate-buffered saline (PBS), *p*H 7.3. Bacterial cell walls were prepared as described earlier (10), and the lipopolysaccharide (LPS) was extracted by the hot phenol-water method (16). All LPS preparations were spun in the ultracentrifuge (105,000 × g, 4 hr) until they were essentially free from material absorbing at 260 nm. If necessary, remaining nucleic acid was removed by treatment with ribonuclease (type XII-A, Sigma Chemical Co., St Louis, Mo.). The chemical structure of these LPS species is shown in Fig. 1. (5-7, 9).

Sera. Antisera against *Salmonella* O-antigens 2, 3, 4, 5, and 9 were prepared in rabbits (2-2.5 kg) of both sexes. Five to eight animals were used for each antigen. For immunization heat-killed bacteria (100 C, 2 hr) suspended in PBS to a concentration of approximately 10^{10} bacteria/ml were used. The animals were given intravenous injections on days 1, 6, and 12 (0.25, 0.5, and 1.0 ml, respectively). They were exsanguinated on day 17 except for the rabbits immunized with *S. senftenberg* (O-antigen 3). These animals were boostered on days 18, 23, and 29 and bled on day 35. Rabbit normal serum (RNS) was from nonimmunized animals. All serum pools were absorbed with heat-killed bacteria of heterologous strains to render them specific for respective O antigens (12). RNS was absorbed with the *Salmonella* strains under study. All sera were inactivated (56 C, 30 min) and absorbed twice with washed, packed sheep erythrocytes prior to testing by the hemagglutination test.

Passive hemagglutination. The hemagglutination experiments were performed in Takatsy microplates (Cooke Engineering Co., Alexandria, Va.), using

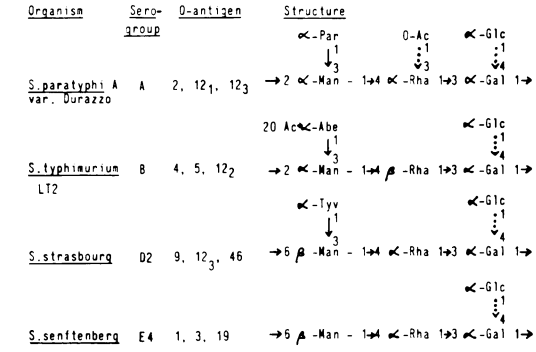

FIG. 1. *Structure of the repeating unit of Salmonella O side-chains. Proposed structure for the O-specific side-chains of group A, B, D_1, and E_4 Salmonella LPS (5–7, 9). A dotted line indicates that not all molecules carry the substituent.*

50-μliter micropipettes. Sheep erythrocytes were sensitized with LPS (63–125 μg/ml) that had been heated for 1 hr at 100 C as described earlier (13). The red cells were washed three times with PBS and used as a 2% suspension. Agglutination was recorded after incubation at room temperature and in the cold for 1 hr and 18 hr, respectively.

Qualitative precipitin analysis. Rabbit antisera were analyzed with a microprecipitin technique (11) using the ninhydrin procedure for nitrogen determination (15). Precipitation was performed in PBS, and controls with LPS alone were always included.

Preparation of enzyme conjugated antiimmunoglobulin. A 0.5-mg amount of antibodies against rabbit immunoglobulin, obtained by immunospecific purification of hyperimmune sheep serum on insolubilized rabbit IgG, was conjugated with 1.5 mg of alkaline phosphatase (calf intestine mucosa, type VII, Sigma Chemical Co., St. Louis, Mo.) by the addition of glutardialdehyde (1–3). Conjugated material was separated from the enzyme and IgG by gel filtration on Sepharose 6 B (Pharmacia AB, Uppsala). The conjugate was stored at 4 C in 5% human serum albumin in 0.05 M tris(hydroxymethyl)aminomethane buffer (*p*H 8.0) containing 0.02% NaN_3.

ELISA. ELISA was performed essentially as described by Engvall and Perlmann (2–4). Disposable polystyrene tubes (11 by 55 mm, Heger Plastics AB, Stallarholmen, Sweden) were incubated with 1 ml of LPS solution (5 μg/ml) in 0.05 M carbonate buffer (*p*H 9.6) containing 0.02% NaN_3, for 3 hr at 37 C. Prior to testing, the tubes were washed three times with 0.9% NaCl containing 0.05% Tween 20. LPS-coated tubes were then incubated with antiserum diluted in PBS with 0.05% Tween 20 and 0.02% NaN_3 (PBSTA) for 6 hr at room temperature. The tubes were then washed as before, and 1 ml of sheep anti-rabbit Ig-alkaline phosphatase conjugate diluted 1:500 in PBSTA was added. After incubation overnight at room temperature, the tubes were washed as above, and 1 ml of a solution of enzyme substrate *p*-nitrophenyl-phosphate (Sigma Chemical Co., St. Louis, Mo.) in 0.05 M carbonate buffer (pH 9.8) containing 10^{-3} M $MgCl_2$ was added. The reaction was developed at room temperature until absorbancy at 400 nm was approximately 1. The reaction was stopped by the addition of 0.1 ml of 1 M NaOH.

RESULTS

Determination of optimal LPS concentration and pH for coating of tubes. Plastic tubes were incubated with varying concentrations of LPS in 0.05 M borate buffer, *p*H 8.0, and tested against a standard dilution of homologous antiserum in the ELISA (Fig. 2). Good sensitization was obtained at about 1 to 5 μg of LPS/ml. At higher concentrations, the absorbance at 400 nm/100 min leveled off, and at still higher concentration there was a decrease in the amount of bound antibody (not shown). All four LPS preparations gave similar sensitization curves. A concentration of 5 μg of LPS/ml at coating was used in the following experiments.

Plastic tubes were incubated with 5 μg of LPS/ml in 0.05 M carbonate buffer (*p*H 9 or 10) and in 0.05 M borate buffer (*p*H 6, 7, and 8) and tested against a standard dilution of homologous antiserum in ELISA. Optimal coating was obtained between *p*H 9 and 10. When tubes were coated with *S. typhimurium* LT2

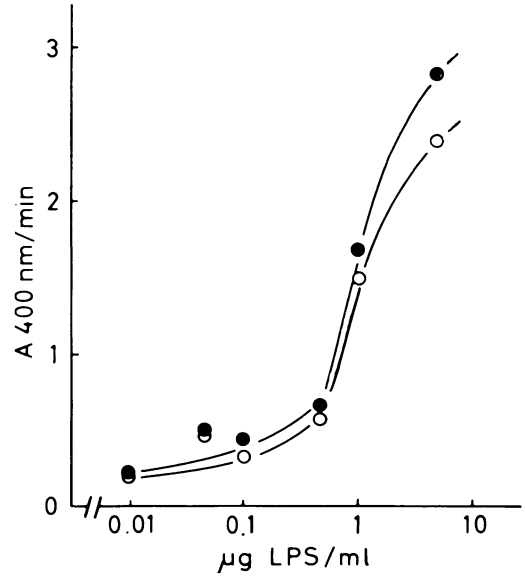

FIG. 2. *Determination of LPS concentration for coating of tubes. Tubes were coated with different concentrations of LPS from S. typhimurium LT2 in 0.05 M borate buffer, pH 8.0, and tested against O4 antiserum (●) and O5 antiserum (○) diluted 1:5,000. The conjugate was diluted 1:500.*

LPS, which contains alkali-labile O-acetyl groups, *p*H 8 was chosen.

Titration of Salmonella O-antigen sera against Salmonella LPS in ELISA. Rabbit antisera against O-antigens 2, 3, 4, 5, and 9 and RNS before and after absorption with homologous bacteria were tested against LPS from *S. strasbourg*, *S. paratyphi* A, var. Durazzo, *S. typhimurium* LT2, and *S. senftenberg* coated on tubes. The antisera were investigated at dilutions 10^{-1} to 10^{-6} by using 10-fold dilution steps. All samples were run in duplicate; the variation in absorbancy at 400 nm between these seldom exceeded 10%. Figure 3 summarizes the result of four such experiments performed under identical conditions. The homologous reaction was always stronger (e.g., antiserum could be diluted 10 to 1,000 times more than any of the heterologous antisera). There was, however, one exception: the O3 antiserum reacted as strongly as the homologous O9 antiserum (anti-$O9^{I}$) against tubes coated with *S. strasbourg* LPS. This cross-reaction was also noted in passive hemagglutination (*see below*). With another O9 antiserum (anti-$O9^{II}$), however, the homologous reaction was stronger (*see* Table 2, only homologous reaction given). LPS from *S. typhimurium* LT2 contains both O-antigens 4 and 5 as a result of incomplete O-acetylation of the abequosyl residue during biosynthesis. Both specificities can, therefore, be demonstrated (Fig. 2 and 3). Figure 3 also shows that RNS contains antibodies to LPS which can be removed by prior absorption with the corresponding LPS. Thus, there is no nonspecific background reaction even at a serum dilution of 10^{-1}.

Titration of Salmonella O-antigen sera against Salmonella LPS by passive hemagglutination. Sheep erythrocytes were optimally sensitized with LPS of the above four *Salmonella* strains (Table 1). For comparison, the results of antiserum titrations in ELISA are also included. The highest dilution of serum, which gives an absorbance of 2.0/100 min at 400 nm, was arbitrarily chosen as the titer in ELISA (Table 1).

There is a good correlation between antibody titers determined by the two methods. Thus with both methods the homologous reaction was, with one exception, always stronger. The exception was the reaction between *S. paratyphi* A LPS and one of the antisera (anti-$O9^{I}$), which was stronger than the homologous anti-O2 reaction in passive hemagglutination. For some of the weak heterologous reactions, there is a lesser correlation between titers determined by ELISA and passive hemagglutination, respectively. These discrepancies may be due to differences in sensitivity for IgG and IgM *Salmonella* antibodies in the two assay systems. In general, ELISA seemed more specific, giving a lower degree of heterologous reactions (compare, for example, *S. senftenberg*).

Quantitative precipitation of Salmonella LPS with Salmonella O-antigen sera. To evaluate the

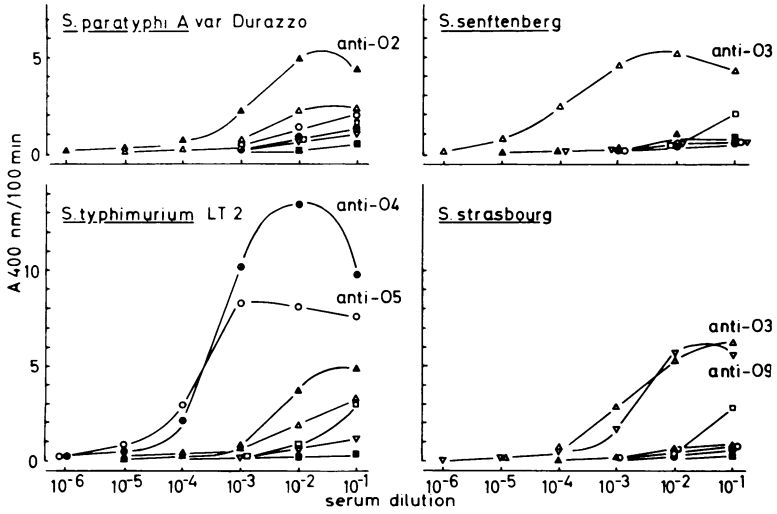

FIG. 3. *Titration of Salmonella O-antigen sera against Salmonella LPS in ELISA. The tubes were coated with 5 μg of LPS per ml from S. paratyphi A variant Durazzo (O-antigen 2), S. senftenberg (O-antigen 3), S. typhimurium LT2 (O-antigens 4 and 5), and S. strasbourg (O-antigen 9), respectively. Symbols: ▲, anti-O2; △, anti-$O3^{I}$; ●, anti-O4; ○, anti-O5; ▽, anti-$O9^{I}$; □, RNS; ■, RNS absorbed with heat-killed bacteria from the Salmonella strains under study. Conjugate dilution, 1:500.*

TABLE 1. *Antibody titers of Salmonella O-antigen sera against Salmonella LPS as determined by ELISA and passive hemagglutination (HA)*

Antigen	Assay method	Titers of antisera						
		Anti-O2	Anti-O4	Anti-O5	Anti-O9[I]	Anti-O3[I]	RNS	RNS abs.[b]
S. paratyphi A var. Durazzo O-antigen 2	ELISA[a]	1:1,000	<1:10	1:10	<1:10	1:200	<1:10	<1:10
	HA	1:100	1:25	1:50	1:200	1:50	1:128	1:64
S. typhimurium LT2 O-antigen 4,5	ELISA	1:500	1:10,000	1:20,000	<1:10	1:100	1:20	<1:10
	HA	<1:10	1:3,200	1:3,200	<1:10	<1:10	1:16	1:8
S. strasbourg O-antigen 9	ELISA	<1:10	<1:10	<1:10	1:1,000	1:2,000	1:10	<1:10
	HA	<1:10	<1:10	<1:10	1:100	1:100	1:16	<1:8
S. senftenberg O-antigen 3	ELISA	<1:10	<1:10	<1:10	<1:10	1:20,000	1:10	<1:10
	HA	<1:10	<1:10	1:25	1:200	1:3,200	1:128	1:64

[a] ELISA titers were defined as the highest dilutions which gave an absorbance of 2.0 in 100 min at 400 nm.

[b] RNS absorbed with heat-killed bacteria from the *Salmonella* strains under study.

sensitivity of ELISA, the amount of precipitating antibody in the different antisera was investigated. Antisera against O-antigens 3, 4, 5, and 9 were precipitated with homologous LPS (Fig. 4). For calculation of the amount of antibody precipitated, values of 2% and 16% for nitrogen in LPS and antibody, respectively, were used. The sera contained between 0.19 and 4.25 mg of specific antibody per ml (Table 2). In addition to the hemagglutination and Widal titers of the antisera, this table also gives the ELISA titers (defined as above) and the amount of specific antibody needed to give an absorbance of 2.0/100 min at 400 nm. As can be seen, 20 to 90 ng was sufficient.

Titration of IgM- and IgG-containing fractions of O3 antiserum in ELISA. Six milliliters of rabbit O3 antiserum (anti-O3[II]) was fractionated by gel filtration on a Sephadex G-200 column (130 by 3.5 cm) by using PBS as eluent. The fractions were pooled (Fig. 5A) and concentrated to the original serum volume. They were then tested in ELISA, passive hemagglutination test, and quantitative precipitation test against homologous LPS (Fig. 5, Table 2). Approximately 70% of the antibodies were found in the IgM-containing fraction as estimated from precipitation data. This fraction also gave the highest hemagglutination and ELISA titers. When the IgM- and IgG-containing fractions were compared in ELISA, approximately three times more IgG was needed to give the same titer (Table 2). Compared to hemagglutination, the IgM-containing fraction was about 10 times more active than the IgG-containing fraction on a weight basis.

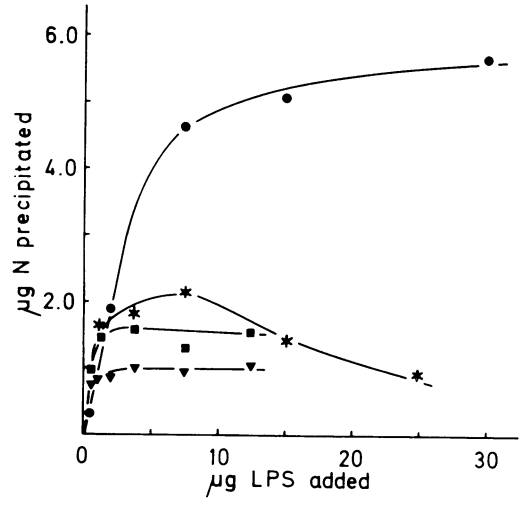

FIG. 4. *Precipitation of Salmonella LPS with homologous O-antigen sera. Symbols: ●, anti-O3[II] (15 μliters) against S. senftenberg LPS; ✳, anti-O9[II] (50 μliters) against S. strasbourg LPS; ■, anti-O5 (20 μliters) against S. typhimurium LT2 LPS; ▼, anti-O4 (30 μliters) against S. typhimurium LT2 LPS. The total volume was 300 μliters.*

DISCUSSION

These studies demonstrate that ELISA is a highly sensitive and specific method for the detection of antibodies to *Salmonella* O antigens.

At slightly alkaline *p*H, LPS is nonspecifically adsorbed to the surface of plastic tubes in sufficient amounts to allow testing for antibodies.

TABLE 2. *Comparative antibody determinations of Salmonella O-antigen sera tested against homologous lipopolysaccharides*

Antiserum	Specific antibody (mg/ml)[a]	Hemagglutination titer	Widal titer	ELISA titer[b]	Antibody giving 1 ELISA titer (ng)
O2	ND[c]	1:100	1:160	1:1,000	
O4	0.19	1:3,200	1:1,280	1:10,000	19
O5	0.47	1:3,200	1:1,280	1:20,000	24
$O9^{II}$	0.25	1:256	1:320	1:5,000	50
$O3^{II}$	4.25	1:1,024	1:320	1:100,000	42
$O3^{II}$ IgM fraction	2.55	1:1,024	ND	1:100,000	26
$O3^{II}$ IgA fraction	0.10	1:128	ND	1:2,000	50
$O3^{II}$ IgG fraction	0.90	1:64	ND	1:10,000	90
$O3^{II}$ Albumin fraction	0.05	1:16	ND	1:1,000	50

[a] Determined by quantitative precipitation.
[b] ELISA titers were defined as the highest dilutions which gave an absorbance of 2.0 in 100 min at 400 nm.
[c] Not done.

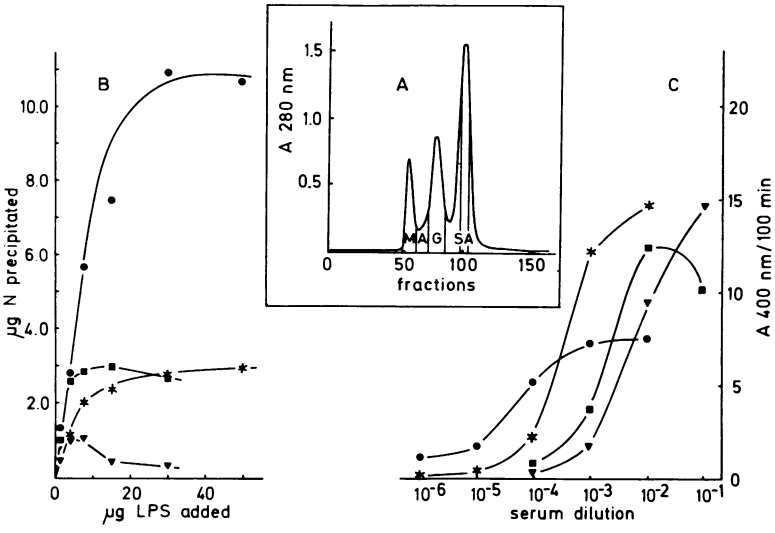

FIG. 5. *Quantitative precipitation and ELISA titration of fractionated O3 antiserum. A, Gel filtration of 6 ml of serum (anti-$O3^{II}$)[10] on a Sephadex G-200 column (130 by 3.5 cm) in PBS. Abbreviations: M, IgM-containing fraction; A, IgA-containing fraction; G, IgG-containing fraction; SA, serum albumin-containing fraction. B, Quantitative precipitation of S. senftenberg LPS with different immunoglobulin fractions of O3 antiserum. Symbols: ●, IgM-fraction (25 μliters); ✳, IgG fraction (15 μliters); ■, IgA fraction (150 μliters); and ▼, serum albumin fraction (250 μliters). The experiments were performed in PBS (total volume, 300 μliters), with the volumes of immunoglobulin fractions given above). C, ELISA titration of O3 antibodies in different immunoglobulin fractions. The tubes were coated with 5 μg of S. senftenberg LPS per ml. The conjugate was diluted 1:500. Symbols as in B.*

Five to ten micrograms of LPS/ml in the incubation mixture was found to be an adequate concentration under the experimental conditions. Alkali-labile O-acetyl groups in LPS (O-antigen 5, *S. typhimurium*) are, furthermore, not significantly destroyed if coating is performed at *p*H 8.0 (Fig. 2). LPS from different strains coated plastic tubes with slightly different efficiency even under standardized conditions. The particular amount of LPS on the tube is, however, not important as long as there is enough LPS to give a significant binding of antibody as detected by bound anti-Ig-enzyme conjugate. Furthermore, variations in LPS concentrations on tubes do not affect the determination of antibody titer significantly.

The ELISA allows specific determination of *Salmonella* antibodies. Homologous reactions were always stronger than heterologous reactions, often by several orders of magnitude; normal rabbit serum gave only weak reactions (Fig. 3). The pattern of reactivity of antisera against different LPS in ELISA was closely similar to that found by passive hemagglutination. For example, the known cross-reaction in hemagglutination between O3 antiserum and heterologous *S. strasbourg* LPS was also found by ELISA (12).

ELISA is more sensitive than passive hemagglutination and Widal agglutination tests. The difference in titers, as determined by ELISA and hemagglutination tests, was by a factor of 10 (Fig. 3; Table 2). Expressed as nanograms of specific antibody, 20 to 90 ng was sufficient to give an absorbance of 2.0/100 min at 400 nm in ELISA (Table 2). It is obvious that at least 10 times lower concentrations of *Salmonella* antibodies can be accurately determined if closer serum dilution steps are used (Fig. 3). The results also show that antibodies of both the IgM and IgG immunoglobulin classes are detected with the antiimmunoglobulin conjugate used in this study. This is expected since the sheep anti-rabbit immunoglobulin serum used to prepare the conjugate reacts with light-chain determinants. With this conjugate, IgM antibodies are detected slightly more efficiently than IgG antibodies in ELISA (Table 2). The difference is by a factor of two to three, as calculated from the amount of antibody in the IgM- and IgG-containing Sephadex G-200 fractions. In hemagglutination, the IgM-containing fraction was approximately 10 times more active than the IgG-containing fraction on a weight basis. Thus, ELISA has the advantage over hemagglutination of also detecting IgG antibodies relatively efficiently. O3 antibodies of IgG class gave a higher maximal uptake level than corresponding IgM antibodies (Fig. 5C). This result, which was obtained with the same batch of LPS-coated tubes, and in excess of conjugate, may indicate that fewer antibodies of IgM class are bound to a given amount of LPS than are IgG antibodies. Alternatively, anti-Ig-enzyme conjugate may be taken up less efficiently on IgM than IgG antibodies.

Furthermore, ELISA may allow the detection and semiquantitation of *Salmonella* O antibodies of different immunoglobulin classes if conjugates are prepared from specific anti-heavy-chain sera.

Finally, under these experimental conditions, the total time to perform an ELISA was 26 to 28 hr. It is, however, possible to decrease the time needed substantially. This may be achieved by shortening the incubation periods with serum and conjugate, respectively, by automatic washing, and by using a device for automatic spectrophotometrical reading.

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LITERATURE CITED

1. Avrameas, S. 1969. Coupling of enzymes to proteins with glutardialdehyde. Use of the conjugates for the detection of antigens and antibodies. Immunochemistry 6:43–52.
2. Engvall, E., K. Jonsson, and P. Perlmann. 1971. Enzyme-linked immunosorbent assay. II. Quantitative assay of protein antigen immunoglobulin G by means of enzyme-labelled antigen and antibody coated tubes (BBA 35 998). Biochem. Biophys. Acta 251:427–434.
3. Engvall, E., and P. Perlmann. 1971. Enzyme-linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin G. Immunochemistry 8:871–874.
4. Engvall, E., and P. Perlmann. 1972. Enzyme-linked immunosorbent assay, ELISA. III. Quantitation of specific antibodies by enzyme-labelled anti-immunoglobulin in antigen coated tubes. J. Immunol. 109:–129–135.
5. Hellerqvist, C. G., B. Lindberg, Å. Pilotti, and A. A. Lindberg. 1970. Structural studies on the O-specific side-chains of the cell-wall lipopolysaccharides from *Salmonella strasbourg*. Acta Chem. Scand. 24:1168–1174.
6. Hellerqvist, C. G., B. Lindberg, Å. Pilotti, and A. A. Lindberg. 1971. Structural studies of the O-specific side-chains of the cell-wall lipopolysaccharide from *Salmonella senftenberg*. Carbohyd. Res. 16:297–302.
7. Hellerqvist, C. G., B. Lindberg, K. Samuelsson, and A. A. Lindberg. 1971. Structural studies on the O-specific side-chains of the cell-wall lipopolysaccharide from *Salmonella paratyphi* A var. Durazzo. Acta Chem. Scand. 25:955–961.
8. Hellerqvist, C. G., B. Lindberg, S. Svensson, T. Holme, and A. A. Lindberg. 1968. Structural studies on the O-specific side-chains of the cell-wall lipopolysaccharide from *Salmonella typhimurium* 395 MS. Carbohyd. Res. 8:43–55.
9. Hellerqvist, C. G., B. Lindberg, S. Svensson, T. Holme, and A. A. Lindberg. 1969. Structural studies on the O-specific side-chains of the cell-wall lipopolysaccharide from *Salmonella typhimurium* LT2. Carbohyd. Res. 9:237–241.
10. Holme, T., A. A. Lindberg, P. J. Garegg, and T. Onn. 1968. Chemical composition of cell-wall polysaccharide of rough mutants of *Salmonella typhimurium*. J. Gen. Microbiol. 52:45–54.
11. Kabat, E. A. 1961. Experimental immunochemistry, 2nd ed. Charles C Thomas, Springfield, Ill.
12. Kauffmann, F. 1966. Enterobacteriaceae. Munksgaard, Copenhagen.
13. Lindberg, A. A., and T. Holme. 1968. Immunochemical studies on cell-wall polysaccharide of rough mutants of *Salmonella typhimurium*. J. Gen. Microbiol. 52:55–65.
14. Neter, E. 1956. Bacterial hemagglutination and hemolysis. Bacteriol. Rev. 20:166–188.
15. Shiffman, G., E. A. Kabat, and W. Thompson. 1964. Immunochemical studies on blood groups XXX. Cleavage of A, B and H blood group substances by alkali. Biochemistry 3: 113–120.
16. Westphal, O., O. Lüderitz, and F. Bister. 1952. Über die Extraktion von Bakterien mit Phenol–Wasser. Z. Naturforsch. 7b:148–155.
17. Widal, F. 1896. Serodiagnostic de la fiévrè typhoide. Bull. Mem. Soc. Hop., Paris 13:561–566.